

Award Number: W81XWH-11-2-0083

TITLE: Designed Proteins as Optimized Oxygen Carriers for Artificial Blood

PRINCIPAL INVESTIGATOR: Ronald L. Koder, Ph.D.

CONTRACTING ORGANIZATION: The City College of New York  
New York, NY 10036

REPORT DATE: February 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. <b>PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.</b>					
1. REPORT DATE February 2014		2. REPORT TYPE Annual		3. DATES COVERED 10 JAN 2013 - 9 JAN 2014	
4. TITLE AND SUBTITLE  Designed Proteins as Optimized Oxygen Carriers for Artificial Blood				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-2-0083	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)  Ronald L. Koder, Ph.D.  E-Mail: koder@sci.ccny.cuny.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  The City College of New York 160 Convent Ave MR419 New York, NY 10031				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT This project concerns the transformation of a de novo designed oxygen transport protein into an effective blood substitute. The protein is less than one-third the size of the human hemoglobin monomer, binds two as opposed to one oxygen-carrying heme cofactor per monomer, and is much more temperature stable than human hemoglobin (1). Therefore the protein promises to have a much higher oxygen capacity per unit weight and per unit volume than human hemoglobin. The stepwise goals of the project are to (A) extend the oxyferrous state lifetime by raising the cofactor reduction potential, (B) to optimize the molecular oxygen binding constant to that of human hemoglobin and (C) to create a crosslinked, stabilized preparation of the optimized protein. In the first two years of the project, Aim B was completed and some progress was made on Aim A, the most difficult Aim. Below we describe year three progress, which includes major advances in Aim A, progress in Aim C, and the additional engineering of a new enzymatic activity which addresses a major problem which has been raised in the literature subsequent to the start of the project.					
15. SUBJECT TERMS Artificial Blood, Blood Substitute					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER (include area code)

## Table of Contents

	<u>Page</u>
Introduction.....	1
Body.....	1
Key Research Accomplishments.....	8
Reportable Outcomes.....	8
Conclusion.....	9
References.....	10
Appendices.....	N/A

## INTRODUCTION

This project concerns the transformation of a de novo designed oxygen transport protein into an effective blood substitute. The protein is less than one-third the size of the human hemoglobin monomer, binds two as opposed to one oxygen-carrying heme cofactor per monomer, and is much more temperature stable than human hemoglobin (1). Therefore the protein promises to have a much higher oxygen capacity per unit weight and per unit volume than human hemoglobin. The stepwise goals of the project are to (A) extend the oxyferrous state lifetime by raising the cofactor reduction potential, (B) to optimize the molecular oxygen binding constant to that of human hemoglobin and (C) to create a crosslinked, stabilized preparation of the optimized protein. In the first two years of the project, Aim B was completed and some progress was made on Aim A, the most difficult Aim. Below we describe year three progress, which includes major advances in Aim A, progress in Aim C, and the additional engineering of a new enzymatic activity which addresses a major problem which has been raised in the literature subsequent to the start of the project.

## BODY

*1. Progress toward Aim 1, increasing the oxyferrous state lifetime.* A critical goal of this work is the extension of the lifetime of the oxygen-bound complex to the minutes time scale. This is important in order to enable oxygen transport throughout the body. In year two, we developed a new model for oxyferrous state lifetimes, including an equation which predicts an O<sub>2</sub>-concentration dependence (2). The important parameters are the dissociation constant of the oxyferrous complex and the outer-sphere oxidation rate of the uncomplexed ferrous protein:

$$(1) \quad \text{Oxidation Rate} = k_{ox} \cdot K_d \cdot \frac{([P] + [O_2] + K_d) - \sqrt{([P] + [O_2] + K_d)^2 - 4[P][O_2]}}{2}$$

(A) *Combinations of the improvements in function – incorporating optimized binding sites.* In year 2 we reported the determination of the oxygen affinities and oxyferrous state lifetimes of a series of proteins which incorporate both the increase in heme reduction potential via internal electrostatics changes, the addition of a bioinformatically determined optimal heme binding site and the transition of the protein scaffold from a homodimer to a single chain four helix bundle. Table 1 demonstrates that the addition of the optimized binding site to both ligating helices of the full chain more than triples the lifetime.

**Table 1.** Oxyferrous lifetime for single chain proteins with the optimal binding site

Protein	ligation	R <sub>air</sub> (s <sup>-1</sup> )	K <sub>d,O2</sub> (mM)	k <sub>ox</sub> (s <sup>-1</sup> mM <sup>-2</sup> )	t <sub>1/2</sub> (s) <sup>*</sup>
HFHF	Original design	0.143±0.005	0.032±0.008	5000±1000	4.84±0.04
HFHF_BM1,3	Sequence optimized	0.04±0.01	0.07±0.01	640±90	17±4

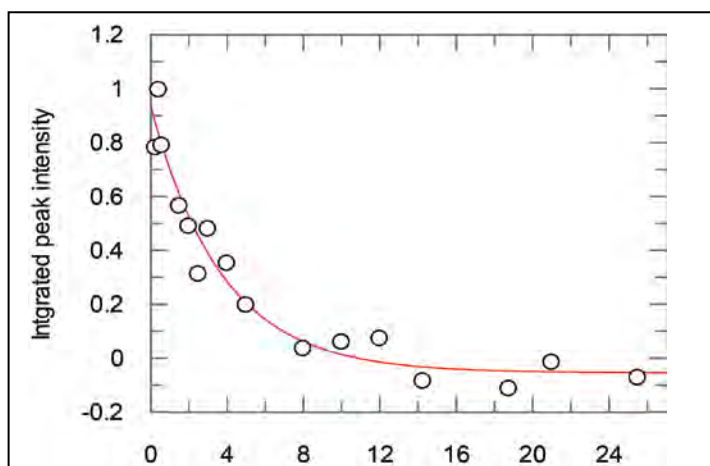
<sup>\*</sup>Oxyferrous state half-life in air (21% O<sub>2</sub>) with 1μM protein heme complex.

**Table 2** | Heme iron ligand on- and off-rates and equilibrium constants in 2 artificial proteins

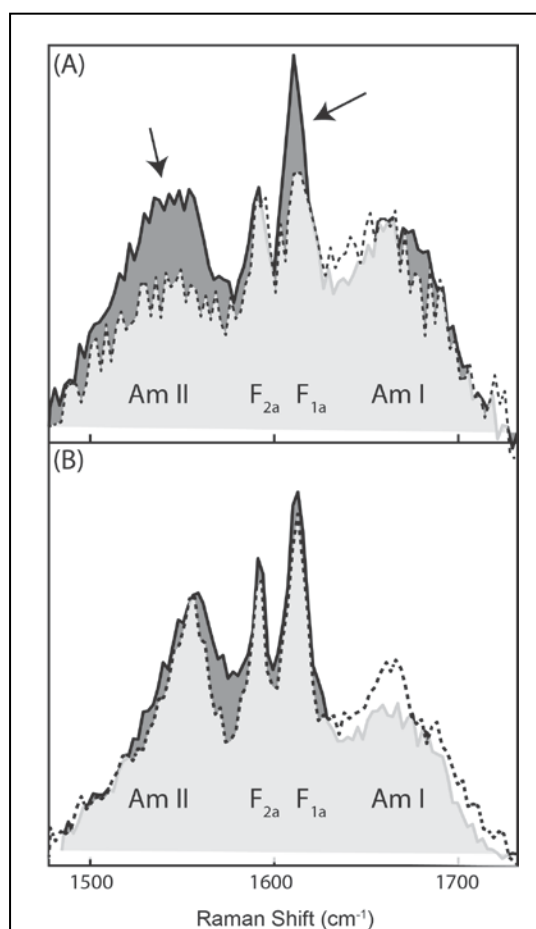
Haem Protein	Gaseous ligand	$k_{\text{gas\_on}}$ ( $\text{mM}^{-1}\text{s}^{-1}$ )	$K_{\text{gas\_off}}$ ( $\text{s}^{-1}$ )	$K_{\text{d\_gas\_pen}}$ ( $\mu\text{M}$ )	$K_{\text{d\_gas\_actual}}$ ( $\mu\text{M}$ )	$k_{\text{his on}}$ ( $\text{s}^{-1}$ )	$k_{\text{his off}}$ ( $\text{s}^{-1}$ )	$K_{\text{A,his}}$
HFHF	CO	430 $\pm$ 150	0.081 $\pm$ 0.001	0.19 $\pm$ 0.7	0.5 $\pm$ 0.4	80 $\pm$ 10	44.5 $\pm$ 0.9	1.8 $\pm$ 0.6
	O <sub>2</sub>	900 $\pm$ 300	3.8 $\pm$ 0.2	4 $\pm$ 2	11 $\pm$ 9			
HFHF_BM1,3	CO	400 $\pm$ 100	0.23 $\pm$ 0.03	0.6 $\pm$ 0.2	3 $\pm$ 2	70 $\pm$ 20	19.6 $\pm$ 0.4	4 $\pm$ 1
	O <sub>2</sub>	640 $\pm$ 180	5.4 $\pm$ 0.6	8 $\pm$ 3	40 $\pm$ 20			

This year we have further characterized the optimized protein to characterize the distal histidine affinity and ligation/detachment rates. These are necessary for publication

We have demonstrated that solvent exchange is more than three-fold slower in the protein with the optimized binding site using NMR-detected amide hydrogen exchange (see Figure 1). We have further, in collaboration with Jason Cooley's research group at the University of Missouri Department of Chemistry, utilized deep UV resonance Raman spectroscopy to compare the hydration of the protein core (Figure 2), a method which we first described in year 2 (3). Thus we have, in two different ways, demonstrated that slowing



**Figure 1.** Hydrogen/deuterium exchange in HFHF\_BM1,3. Line drawn is a fit with an exponential function with a rate constant of  $0.27 \text{ s}^{-1}$ , a value more than three-fold slower than that observed for HFHF.

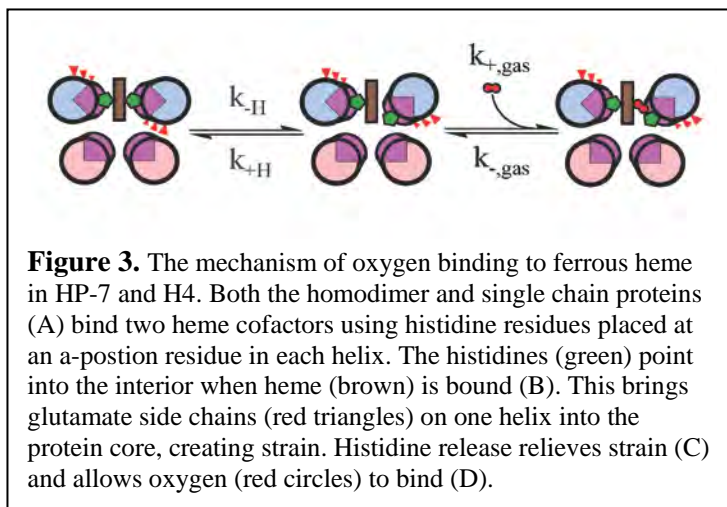


**Figure 2.** dUVRR spectra of the oxidized (Solid line-gray filled) versus reduced (dashed -white filled) HFHF (Panel A) and HFHF\_BM1,3 (Panel B) hemoproteins.

water penetration increases the oxyferrous state lifetime. We have submitted one paper and one patent on this, and another paper is currently under preparation.

*Combining optimized heme binding with electrostatic optimization.* In year two, we worked to elevate the reduction potential of the bound heme in the protein. One method attempted was to remove the three glutamic acid side chains which lay on the outside of the pseudo-symmetric ligating helix (see Figure 3). However, as the exact topology of the four helix bundle is not known, it is impossible to determine which of the two sets of three B-position glutamates lies on the outside and which lie on the inside. Thus we deleted both sets of glutamates, one in each protein.

We determined that one of the deletions, HFHF\_1\_3A\_BM1,3, has an oxyferrous state lifetime more than two-fold longer than any other protein measured (see Table 3). The original intent was to increase the lifetime by increasing the reduction potential. However, this year we determined that the reduction potentials are the same, within error, as the protein with all the glutamates intact (see Table 3). The current thinking is that the increase in lifetime is a result of decreased dynamics in the protein decreasing the rate and magnitude of water penetration.



**Table 3**

Protein	ligation	$E_{\text{mid}}$ (mV)	$R_{\text{air}}$ (s <sup>-1</sup> )	$K_{\text{d,O}_2}$ (mM)	$k_{\text{ox}}$ (s <sup>-1</sup> mM <sup>-2</sup> )	$t_{1/2}$ (s)*
HFHF_BM1,3	Sequence optimized	-297	0.04±0.01	0.07±0.01	640±90	17±4
HFHF_1_3A_B M1,3	1 <sup>st</sup> helix side chain optimized	-293	0.016±0.003	0.024±0.002	740±70	43±8
HFHF_3_3A_B M1,3	3 <sup>rd</sup> helix side chain optimized	-307	0.04±0.01	0.19±0.04	340±50	18±6

\*Oxyferrous state half-life in air (21% O<sub>2</sub>) with 1μM protein heme complex.

2. Progress toward Aim 2, manipulating the oxygen affinity. The effective oxygen affinity of the hexacoordinate hemoglobin is directly affected by competition from the distal histidine. The on-rate at high [O<sub>2</sub>], instead of being second-order with the protein concentration, becomes rate-limited by the distal histidine off-rate. Since the O<sub>2</sub> off-rate is unaffected by the distal histidine, the net effect is to increase the O<sub>2</sub> dissociation constant (4):

$$(2) \quad K_{d,O_2} = K_{d,O_2,pent}(1 + K_{A,His})$$

Where  $K_{d,O_2,pent}$  is the intrinsic pentacoordinate binding constant in the absence of distal histidine interference and  $K_{A,His}$  is the unimolecular association constant of the distal histidine.

The presence of this histidine thus serves to decrease oxygen affinity, and gaining the ability to manipulate this histidine ligand affinity enables the concomitant manipulation of gaseous ligand affinity. One possible method is to protonate the distal histidine, therefore weakening distal histidine binding by proton competition:

$$(3) \quad K_{A,His,eff} = \frac{K_{A,His}}{1 + \frac{[H^+]}{K_a}}$$

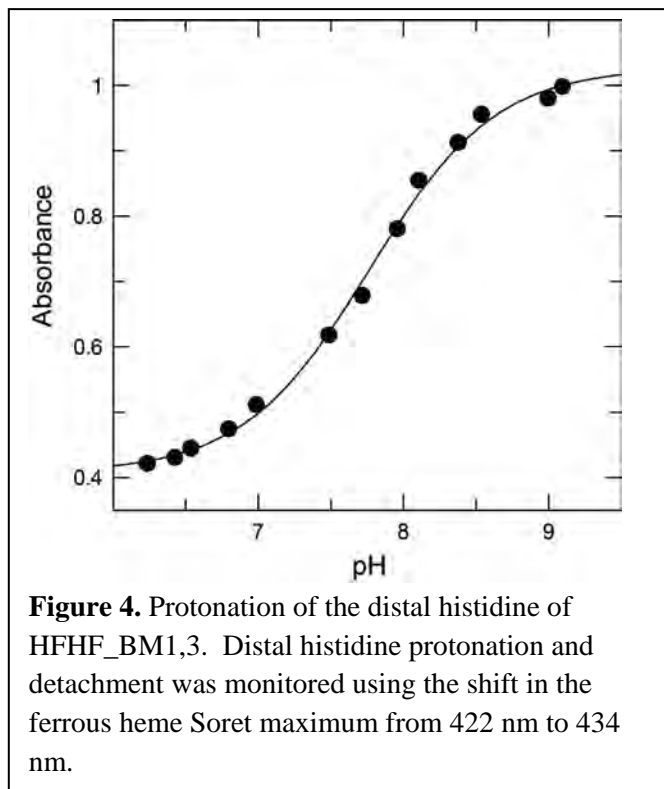
Attempts to do this by lowering the pH in earlier versions of the protein such as HP7 and H4 were unsuccessful, presumably due to further protonation of the proximal histidine ligand. This resulted in the release of the bound

heme. However, in year 2 we

demonstrated that the heme cofactor binding affinity is more than 20-fold higher in the binding site-optimized HFHF\_BM1,3 protein. As Figure 4 demonstrates, in this protein the distal histidine can be protonated without heme detachment. The pK<sub>a</sub> for the distal histidine is 7.9±0.1. Given that the distal histidine K<sub>A</sub> is 4 at pH 9 (Table 1), we can calculate using Eqn. 3 that at pH 7.4 (physiological pH) the K<sub>A</sub> would decrease to 1.6, thus increasing O<sub>2</sub> affinity by a similar factor of 2.5, from a K<sub>d</sub> of 11 μM to one of 4.8 μM.

The lack of competition by histidine is predicted to speed gaseous ligand binding at high oxygen concentration, transforming binding into a single second order process from a two-component process consisting of histidine ligand detachment followed by oxygen binding. This should reduce the fraction of protein which rapidly oxidizes upon exposure to oxygen via an outer-sphere process in which the bis-histidine-ligated ferrous heme iron donates an electron, forming superoxide. Experimental testing of this hypothesis are currently underway.

One exciting possibility is that histidine protonation occurring at this pK<sub>a</sub> offers us a mechanism to recreate the **Bohr effect** in our designed protein, resulting in a protein with a pH-



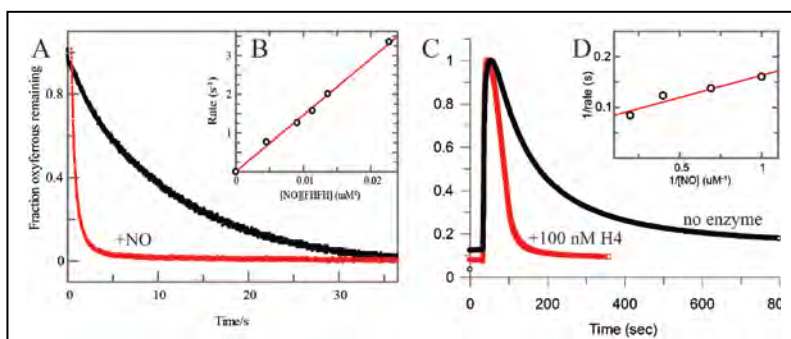
**Figure 4.** Protonation of the distal histidine of HFHF\_BM1,3. Distal histidine protonation and detachment was monitored using the shift in the ferrous heme Soret maximum from 422 nm to 434 nm.

dependent oxygen affinity. This is a critical property in human blood that is thus far not been recreated in a synthetic blood substitute.

3. Nitric oxide dioxygenase activity in this series of proteins. One question that arose during the ARO Blood Substitutes meeting concerned the level of nitric oxide dioxygenase activity in these proteins. Despite their role in oxygen transport, human hemoglobins and myoglobins also display a low level of NOD activity themselves (5, 6). For this reason, blood substitutes composed of crosslinked preparations of bovine and human hemoglobin have been shown to be partially responsible for increased rates of cardiac arrest in clinical trials due to vasodilation and vasoconstriction induced by unwanted nitric oxide transport and dioxygenation (7, 8). It is important to learn how to reduce or eliminate NOD activity in these therapeutics without adversely affecting oxygen binding and transport.

NO Dioxygenase activity in H4. We felt that the bound oxygen in H4 represents an activated oxygen which might be capable of performing NOD chemistry. To determine whether the oxyferrous state of the heme domain reacts with NO (step 2, Figure 3B), we created a mutant form of the single chain diheme domain, termed HFHF, which deleted the proximal heme binding site. We then formed the heme complex, reduced it, and in a double-mixing stopped flow experiment mixed it first with oxygen and then with NO. In the absence of NO the lifetime of the oxyferrous state is >10s. Addition of NO causes rapid oxidation, and the oxidation rate is linearly dependent on the applied NO concentration - consistent with a collisional reaction between NO and the oxyferrous protein-heme complex with a second order rate constant of  $0.15 \text{ s}^{-1}\text{mM}^{-1}$  (Figure 6A and B). Analysis of the reaction products formed, under reaction conditions at which >90% of the products were the result of enzymatic activity, using the nitrate reductase assay of Gilliam *et al.* (9) demonstrated that all of the nitric oxide, within error, that was added to the reaction was oxidized to nitrate anion (not shown).

In the natural hexacoordinate hemoglobins, re-reduction of the oxidized heme (step 3, Figure 3B) occurs via an unidentified reductase protein. For *in vitro* steady-state kinetic examination of full enzymatic turnover the *E. coli* flavoprotein NADP:ferredoxin reductase (FdR) in combination with an NADPH recycling system is used as the electron source (10) and NO concentrations are measured

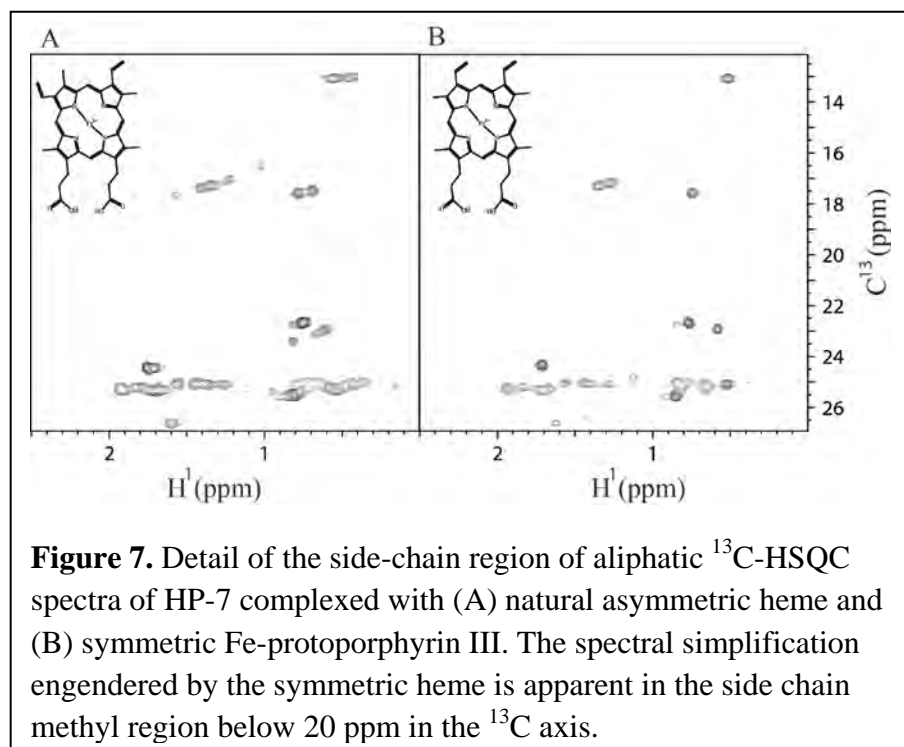


**Figure 6.** Reactions of H4 with NO. (A) Stopped flow analysis of the oxyferrous lifetime in the absence (●) and presence (●) of NO. (B) Inset: replot of the NO dependence of the oxidation rate. (C) Steady state kinetic acceleration of NO dioxygenation by 1  $\mu\text{M}$  H4 and the NADP:ferredoxin reductase NADPH recycling system. (D) Double reciprocal replot of the [NO] dependence of the catalytic rate.

using an NO electrode (5). As figure 6C and D demonstrates, HFHF with one heme bound is catalytically active, with a  $k_{\text{cat}}$  of  $13 \text{ s}^{-1}$  and a  $K_M$  of  $1.5 \text{ }\mu\text{M}$  under conditions of 4%  $\text{O}_2$ , 2mM NADH and 100 nM FdR - conditions saturating for electron donation for neuroglobin and cytoglobin (11, 12). As we have not yet determined the  $K_M$  for  $\text{O}_2$  or looked at whether FdR-based reduction is rate limiting, these numbers are preliminary, and the true  $k_{\text{cat}}$  may be even higher. Control reactions run eliminating oxygen demonstrated that HFHF exhibits no detectable NO reductase activity.

4. Progress toward Aim 3, forming a crosslinked heme protein nanoparticle. The goal of this project is to create a cross-linked preparation of this oxygen transport protein while retaining function. As most chemical crosslink agents react with specific amino acid side chains, it is critical to determine the three-dimensional structure of the protein in order to strategically place these residues.

*NMR Structural analysis – the pathway to a structurally specific protein complex.* Attempts at solving the solution structures of the both original  $\text{O}_2$  transport protein HP7 and its single chain variant H4 were unsuccessful. In both cases the backbone resonances could be assigned, but the side chain resonances were too degenerate to allow resonance assignments (13). One problem was the result of the asymmetric nature of the heme cofactor. The lack of a structurally complementary heme binding site means that the heme can insert in two different orientations, as is observed in some natural systems (14). As Figure 7 demonstrates, the use of the symmetric

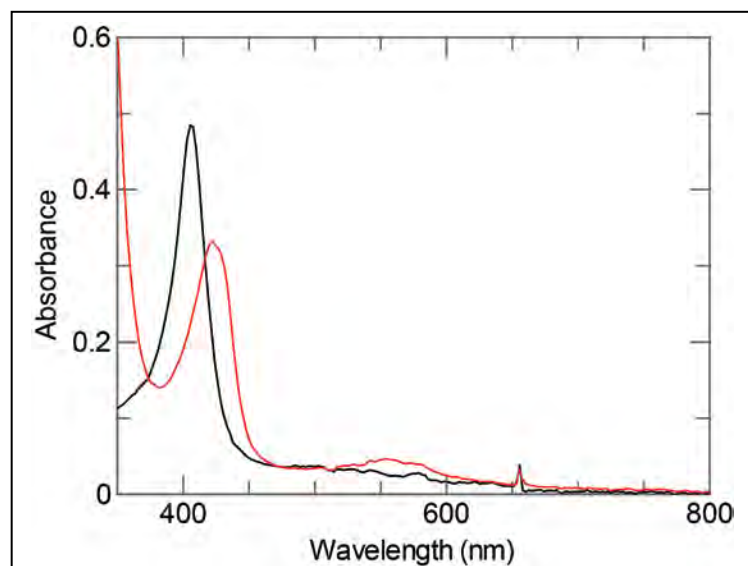


heme analogue Fe-protoporphyrin III greatly reduces side chain disorder in these proteins. Even with the symmetric heme, the side chains of H4 could not be assigned due both to the degenerate nature of the protein sequence (all 4 helices have the same sequence, and each helix is a repeat of the same heptad three times with minor modification for heme binding (15)) and due to side chain

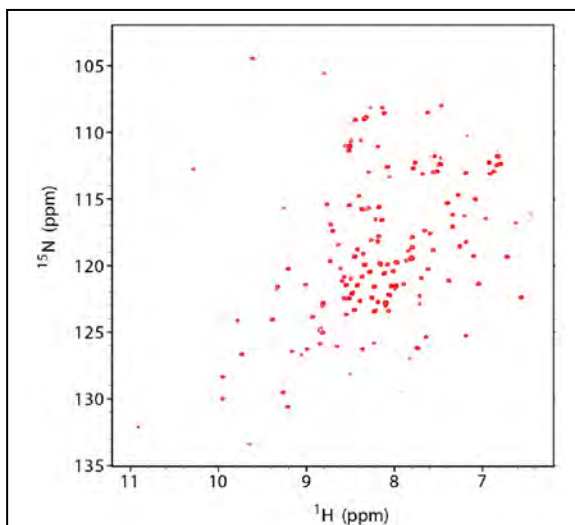
mobility induced by poor core packing around the heme cofactors.

The heme binding sites in HP-7 and H4 are simply composed of histidine residues placed in the proper locations the hydrophobic core. There was no effort made at optimizing a packing interface. Perhaps for this reason, these proteins have eluded full structural analysis – crystallization attempts have been unsuccessful and there is not sufficient chemical shift dispersion in the side chains to allow structural resolution by NMR. We thought the latter might be due to side chain mobility induced by poor core packing around the heme cofactors.

To improve this we utilized our recently published bioinformatic analysis of heme binding proteins which examined the rotamer distribution for helical histidines bound to heme cofactors (16). This analysis revealed strong consensus sequences which differed greatly for each rotamer - different ligand histidine conformations place the heme in contact with different residues on its helix. Use of a histidine specific label enabled us to demonstrate that histidine residues in both sites of H4 are in the t-73 rotamer (not shown). Insertion of the optimal t-73 residues into H4, resulting in the single chain protein BM4, results in a diheme protein with 20-fold tighter binding than H4 in both the oxidized and reduced state. Unlike H4, the apoprotein is structured, and addition of heme cofactors results in a protein which displays significantly higher chemical shift dispersion (Figure 4). Observable backbone and sidechain resonances of



**Figure 9.** Oxidized and reduced spectra of a suspension of crosslinked H4.



**Figure 8.**  $^{15}\text{N}$ -HSQC spectrum of ferrous diheme HFHF\_BM1,3.

HFHF\_BM1,3 have been assigned. Currently, 4D HSQC-NOESY-HSQC experiments, both  $^{13}\text{C}$ - $^{13}\text{C}$  and  $^{15}\text{N}$ - $^{13}\text{C}$ , are being collected and assigned in order to determine the full 3D structure. Determination of this structure would be a significant scientific achievement – the first full structure of a designed, cofactor-containing protein.

(D) *Crosslinking.* Given the large conformational change necessary for oxygen transport function, a full structural analysis is necessary to preclude the possibility of ‘locking

down' the closed conformation of the heme-protein complex via cross-linking. However, a preliminary crosslink of unaltered H4 was attempted to determine whether the crosslinking process interfered with heme complex assembly.

HFHF\_BM1,3 was crosslinked using glutaraldehyde as has been published previously for myoglobin (17). Particles were purified and the protein was shown to remain in a folded and functional state by absorption spectroscopy – both oxidized and reduced spectra were that of a bis-histidine bound heme (Figure 9). As expected, this material does not form an oxyferrous state, instead it just oxidizes upon exposure to molecular oxygen (not shown). Once a full structure is determined, glutaraldehyde-reactive surface-exposed lysine residues on the rotating helix (Figure 3) will be mutated to glutamine, and function will likely be recovered.

## KEY RESEARCH ACCOMPLISHMENTS

1. We have experimentally determined that both crosslinking monomers of HP-7 to form H4 and adding a sequence-optimized heme binding site increase the oxyferrous state lifetime by reducing water penetration and not by electrostatics.
2. We have demonstrated a Bohr effect in oxygen binding caused by the protonation of the distal histidine at a  $pK_a$  of 7.4. This is a critical part of natural hemoglobin function and is a first in the artificial blood field.
3. We have assessed the nitric oxide dioxygenase activity of H4, and shown that the rates of reaction are similar to that of human hemoglobin.
4. We have demonstrated that symmetric heme compounds greatly simplify the NMR spectra of these protein complexes, and have made significant progress toward a full solution structure.
5. We have crosslinked the best performing protein thus far and demonstrated that it retains heme binding ability, but not the ability to form an oxyferrous state.

## REPORTABLE OUTCOMES

### *Manuscripts*

1. Brown, M.C., Mutter, A.C., Koder, R.L., JiJi, R.D., Cooley, J.W. (2013). Direct quantification of persistent  $\alpha$ -helical content and discrete types of backbone disorder during a molten globule to ordered peptide transition. *J. Raman Spect.* 44:957-962
2. Zhang, L., Brown, M.C., Mutter, A.C., Cooley, J.W., Koder R.L. (2014). Dynamic Water Penetration Limits the Oxyferrous State Lifetime of an Artificial Oxygen Transport Protein. Submitted
3. Everson, B.H., French, C.H., Mutter, A.C., Nanda, V., Koder, R.L. Hemoprotein Design Using Minimal Sequence Information. In preparation
4. Anderson, E., Zhang, L., Brisendine, J., Hargrove, M.S., **Koder, R.L.**, Nitric Oxide Dioxygenase Activity in an Artificial Hemoprotein. In preparation
5. French, C.H., Everson, B.H., Mutter, A.C., **Koder, R.L.** Protein Supercharging

Modulates Cofactor Binding by Altering Internal Electric Fields. In preparation

*Presentations.*

1. University of Missouri Dept. of Chemistry: 'Engineering Artificial Protein Function' Columbia, MO, May 2014
2. Johns Hopkins University Dept. of Chemistry: 'Engineering Artificial Protein Function' Baltimore, MD, May 2014
3. New York Area Nanotechnology Discussion Group, 'Protein Design and Self Assembling Metamaterials', New York, NY April 2014
4. Royal Society International Scientific Seminar on Design, Engineering and Evolution of Biomolecular Components, 'Biomaterial Design Using the Inside-Out Algorithm', Bristol, UK, Nov 2014

## CONCLUSIONS

We have made further progress in understanding the structural and dynamic engineering features which underlay oxygen transport function, finding that water penetration dominates over electrostatics in controlling oxyferrous state lifetimes. Importantly, we have recreated a key aspect of human hemoglobin function, the 'Bohr effect', in which small changes in solution pH induce large changes in oxygen affinity. We have determined that the nitric oxide dioxygenase activity of the protein is equal to or less than that of human hemoglobin. Crosslinking attempts on the protein demonstrate that a full three-dimensional structure of the protein is necessary in order to create a functional oxygen transport particle. Furthermore, the employment of symmetric cofactors has engendered a significant increase in the quality of the NMR spectra of the protein, and enabled critical progress on the assignment of the protein side chains.

*Why is this important or "So what":* The Bohr effect results in a high oxygen affinity in the majority of the circulatory system and a weakened affinity in the low pH environment of capillaries, promoting oxygen release in the extremities. It is a fundamental aspect of hemoglobin function that has yet far been unreproduced in any cross-linked natural blood preparation. The incorporation of this aspect of hemoglobin function is thus an important milestone in synthetic blood engineering.

Nitric oxide dioxygenase activity has been a major obstacle to the creation of a blood substitute. Unwanted elimination of nitric oxide has resulted in cardiac complications in clinical trials. It is important that the artificial protein have a lower level of this activity.

We have shown the three dimensional structure of the protein to be critical for the creation of a cross-linked preparation of the protein which retains function. The utilization of a symmetric heme analogue has removed a major barrier to the determination of this structure.

## References

1. Koder RL, *et al.* (2009) Design and engineering of an O<sub>2</sub> transport protein. *Nature* 458:305-309.

2. Zhang L, Andersen EME, Khajo A, Magglio RS, & Koder RL (2013) Dynamic factors affecting gaseous ligand binding in an artificial oxygen transport protein. *Biochemistry* 52:447-455.
3. Brown MC, Mutter AC, Koder RL, Ji Ji RD, & Cooley JW (2013) Observation of persistent  $\alpha$ -helical content and discrete types of backbone disorder during a molten globule to ordered peptide transition via deep-UV resonance Raman spectroscopy. *J. Raman Spectrosc.* 44(7):957-962.
4. Kakar S, Hoffman FG, Storz JF, Fabian M, & Hargrove MS (2010) Structure and reactivity of hexacoordinate hemoglobins. *Biophys. Chem.* 152(1-3):1-14.
5. Smagghe BJ, Trent JT, 3rd, & Hargrove MS (2008) NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction in vivo. *PLoS One* 3(4):e2039.
6. Eich RF, *et al.* (1996) Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* 35(22):6976-6983.
7. Chen JY, Scerbo M, & Kramer G (2009) A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics (Sao Paulo)* 64(8):803-813.
8. Varnado CL, *et al.* (2013) Development of recombinant hemoglobin-based oxygen carriers. *Antioxid Redox Signal* 18(17):2314-2328.
9. Gilliam MB, Sherman MP, Griscavage JM, & Ignarro LJ (1993) A spectrophotometric assay for nitrate using NADPH oxidation by *Aspergillus* nitrate reductase. *Anal Biochem* 212(2):359-365.
10. Hayashi A, Suzuki T, & Shin M (1973) An enzymic reduction system for metmyoglobin and methemoglobin, and its application to functional studies of oxygen carriers. *Biochim Biophys Acta* 310(2):309-316.
11. Brunori M, *et al.* (2005) Neuroglobin, nitric oxide, and oxygen: functional pathways and conformational changes. *Proc Natl Acad Sci U S A* 102(24):8483-8488.
12. Trent JT, Watts RA, & Hargrove MS (2001) Human neuroglobin, a hexacoordinate hemoglobin that reversibly binds oxygen. *J. Biol. Chem.* 276(32):30106-30110.
13. Koder RL, *et al.* (2006) Native-like structure in designed four helix bundles driven by buried polar interactions. *J. Am. Chem. Soc.* 128(45):14450-14451.
14. Lamar GN, Budd DL, Viscio DB, Smith KM, & Langry KC (1978) Proton Nuclear Magnetic-Resonance Characterization of Heme Disorder in Hemoproteins. *Proc. Natl. Acad. Sci. U. S. A.* 75(12):5755-5759.
15. Huang SS, Koder RL, Lewis M, Wand AJ, & Dutton PL (2004) The HP-1 maquette: From an apoprotein structure to a structured hemoprotein designed to promote redox-coupled proton exchange. *Proc. Natl. Acad. Sci. U. S. A.* 101(15):5536-5541.
16. Negron C, Fufezan C, & Koder RL (2009) Helical Templates for Porphyrin Binding in Designed Proteins. *Proteins* 74:400-416.
17. Harris DR & Palmer AF (2008) Modern Cross-Linking Strategies for Synthesizing Acellular Hemoglobin-Based Oxygen Carriers. *Biotechnology Progress* 24(6):1215-1225.